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TITLE: A Role for MEK-Interacting Protein 1 in Hormone Responsiveness of ER Positive Breast Cancer Cells

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14. ABSTRACT
Our results have revealed a critical and previously unreported role for the small scaffold protein MP1 in the survival of ER-positive, but not ER-negative breast cancer cell lines. Blocking MP1 expression using siRNA leads to apoptotic cell death in ER-positive MCF-7, LCC9 and T47D cells, but not in ER-negative MDA-MB-231, SKBr3 and BT-549 cells. Overexpression of MP1 may also be toxic to ER-positive cells, since MCF-7 derivatives expressing ectopic MP1 are unstable and rapidly lose expression of the transfected MP1 gene upon passage. The mechanism by which MP1 affects survival of ER-positive breast cancer cells is not known, but may be the result of alterations in ER levels and/or functions. In support of this hypothesis, MP1 knockdown leads to decreased ER levels, and MP1 overexpression to increased ER levels. Together, out data strongly suggest that MP1 could provide a novel target for the treatment of ER-positive breast tumors. Future experiments will investigate the mechanism(s) by which MP1 regulates survival of ER-positive breast cancer cells, and those by which it regulates ER levels and function.

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Introduction

Our original proposal presented data characterizing an interaction between estrogen receptor alpha (ER) and the small scaffold protein Mek Partner-1 (MP1) in human breast cancer cell lines. The aims of this proposal were **1: To test the hypothesis that MP1 is required for ER function and proliferation in human breast cancer cells, and 2: To characterize the subcellular localization and protein composition of ER/MP1 complexes.** In our first annual report, we presented data showing that inhibition of MP1 expression using siRNA led to cell death in ER-positive MCF-7 cells but not in ER-negative MDA-MB231 cells, suggesting a specific requirement for MP1 expression in ER-positive cells. These results have now been extended to additional cell lines, and the mechanism of cell death has been investigated. Our year 1 report also described the difficulties that we experienced expressing MP1 in stably transfected cell lines, and in carrying out proposed co-immunoprecipitation experiments. Our progress in these experiments is also reported below.

Body: Progress on Each Task in Approved Statement of Work.

Task 1: Test hypothesis that MP1 expression is required for ER's transcriptional activity and proliferation of human breast cancer cells.

In year 1 of this project we observed that knockdown of MP1 led to cell death in ER-positive MCF-7 cells within 48 h (previous progress report). We have now extended this analysis to two additional ER-positive cell lines, MCF-7/LCC9 (LCC9), an MCF-7 derivative with acquired resistant to both ICI 182,780 and Tamoxifen, and T47D. MCF-7 was the most sensitive of the three cell lines, with the majority of cells detaching from the plates by 48 h, and with 84% of detached cells and 27% of attached cells being scored as dead by trypan blue exclusion. Neither LCC9 nor T47D exhibited the same extent of cell detachment as MCF-7; however, both showed a significant increase in cell death in cells transfected with MP1 siRNA compared to control siRNA. In LCC9, 75% of detached cells and 32% of attached cells were non-viable, and in T47D 80% of detached cells and 38% of attached cells were non-viable (Figure 1). In our year 1 report, we showed that knock-down of MP1 did not result in cell death in MDA-MB-231, an ER-negative human breast cancer cell line. We have now extended these results to two additional ER-negative cell lines, BT-549 and SK-Br3. In each case, there was no significant difference in cell death between control siRNA and MP1 siRNA transfected cultures (Figure 2). These results strongly suggest that MP1 is required for the survival of ER-positive, but not ER-negative, breast cancer cell lines, and may therefore provide a novel target for ER-positive breast tumors.

In our first annual report, we proposed to investigate if the cell death that we detected by trypan blue exclusion was the result of apoptosis. To address this issue, we assayed for PARP cleavage, an indicator of apoptosis, in cells transfected with MP1 siRNA or control siRNA. Transfection with two independent MP1 siRNAs induced PARP cleavage in MCF-7 cells (Figure 3A), and this effect was blocked by the pan-caspase inhibitor v-VAD-FMK (Figure 3B). No PARP cleavage was detected in MDA-MB 231 cells (Figure 3A), consistent with the lack of death observed in this cell line. The increase in apoptosis in MCF-7 cells was confirmed by Annexin V staining, where a significant increase in Annexin V positive cells was detected in MP1 siRNA vs. control siRNA transfected cells (data not shown). To evaluate the effects of MP1 knockdown

on ER's transcriptional activity, MCF-7 cells were co-transfected with control siRNA or MP1 siRNA, along with both an ERE-Luc reporter and β-gal control gene, and incubated in medium lacking E2 (IMEM plus 5% charcoal stripped serum) for 24 h. Cells were then treated with vehicle or E2, and both luciferase and β-gal activities were measured. As shown in Figure 4A, knockdown of MP1 resulted in an approximate 2-fold decrease in E2-induced ER activity on an ERE containing promoter. Western blotting experiments demonstrated that ER levels decrease in MP1 siRNA-transfected cells (Figure 4B), providing a possible mechanism for the decreased transcriptional activity.

Task 2: Determine the subcellular localization of ER/MP1 complexes.

In our original application, we proposed to identify the subcellular localization of ER/MP1 complexes by isolating various fractions (membrane, cytosol, nucleus, etc.) and then analyzing these fractions for ER/MP1 complexes by co-immunoprecipitation experiments. As described in our first annual report, we experienced technical difficulties completing these experiments because we were unable to immunoprecipitate endogenous ER/MP1 complexes with commercial antibodies. We have not been able to resolve these difficulties, but have tried to address the issue using transfected cell lines containing epitope tagged proteins. These experiments are described under Task 3 below. We have also carried out cell fractionation experiments to examine the subcellular localization of MP1 and ER. As expected, ER was predominantly nuclear, with a small amount in the cytosol. Previous subcellular localization using GFP-MP1 fusion proteins have indicated that MP1 is primarily localized in late endosomes (1). In preliminary biochemical fractionations have detected some MP1 in nuclear fractions (data not shown). However, given the small size of endogenous MP1 (approximately 13.5 kilodaltons), this could be due to diffusion through nuclear pores. Experiments in the coming year will more fully investigate the subcellular localization of endogenous MP1.

Task 3: Purify ER/MP1 complexes by sequential affinity purification and examine complex components by Western blotting.

As described in last year's report, we originally proposed to carry out these experiments in cell lines with inducible Flag-MP1 expression using the AP21967 (AP) - inducible gene expression developed by ARIAD (2). Surprisingly, when a Flag-MP1 construct was expressed using this system, expression was constitutive in both stably transfected cell lines and in cells transiently infected with a retrovirus construct. In addition, we have observed a loss of Flag-MP1 expression in stably transfected or infected MCF-7 derivatives with cell passage, suggesting a toxic effect of ectopic MP1 expression. Despite these difficulties, we have proceeded to conduct the originally proposed co-immunoprecipitation experiments. An early passage stably transfected cell line expressing Flag-MP1 (clone 5) was incubated in medium containing charcoal stripped serum (CSS), CSS+E, CSS+Tam and CSS+ICI. Cell lysates were prepared and immunoprecipitated with anti-Flag antibodies. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with both anti-MP1 and anti-ER antibodies. As shown in Figure 5A, the Flag-MP1 was efficiently precipitated and detected. However, no ER was detected in the IP pellets, although it was readily detected in the cell lysates. The ER/MP1 co-immunoprecipitation

experiments have also been carried out in reverse using an inducible Flag-ER cell line (Figure 5B). In this case, all treatments were carried out in the absence and presence of AP to induce Flag-ER expression. Similar to the results with the Flag-MP1 cell line, Flag-ER was efficiently precipitated using the anti-Flag antibody, but no MP1 was detected in the IP pellet although it was readily detected in the cell lysates.

If only a small percentage of the ER and MP1 in cells are present in complexes with each other, the sensitivity of the co-IP experiments in Figure 5 might not be sufficient to detect such complexes. One way to overcome this limitation might be to over express both proteins. We therefore performed co-transfection experiments in which human 293 cells (which do not express ER) were transfected with expression vectors encoding Flag-MP1 and/or ER, or the appropriate empty vector controls. The transfected cells were incubated in medium containing CSS, then treated with vehicle or E for 3 h. Cell lysates were then prepared and immunoprecipitated with anti-Flag antibody. Both IP pellets and total cell lysates were analyze for ER and MP1 levels by immunoblotting. As shown in Figure 6, Flag-MP1 was efficiently expressed and precipitated in these experiments, but ER was not detectable in the IP pellets although it was present in cell lysates. Interestingly, however, co-transfection with MP1 did affect ER expression in these experiments. As shown in Figure 6, E2 treatment decreases ER levels in the absence (lane 3), but not the presence (lane 2), of co-transfected MP1. This experiment has been repeated a second time with similar results (not shown). The ability of co-transfected MP1 to increase ER levels is consistent with our finding that inhibition of MP1 expression with siRNA leads to decreased ER levels (Figure 4), and suggests that MP1 plays a role in regulating ER levels. This role is likely post-transcriptional, since the ER gene in Figure 6 is expressed from a CMV promoter.

In summary, we have successfully expressed and immunoprecipitated Flag-MP1 and Flag-ER in transfected cell lines, but have been unable to demonstrate co-immunoprecipitation of the two proteins. At this point we do not have an explanation for the difference between these results and those that were presented in our original grant application, other than a change in personnel. We will continue to try to identify conditions where we can demonstrate a physical complex containing these two proteins by altering lysis buffers, wash conditions, etc.

Task 4: Identify novel components of ER/MP1 complexes by mass spectrometry.

The experiments described in this task are dependent on purifying ER/MP1 complexes. As described under Task 3, we have been unable to accomplish this to date.

Key Research Accomplishments:

- Demonstrated that MP1 expression is required for survival of ER-positive cell lines MCF-7, MCF-7/LCC9 and T47D, but not ER-negative cells MDA-MB-321, BT-549 and SK-BR3.
- Established mechanism of cell death as apoptosis.
- Demonstrated that knock-down of MP1 leads to decreased ER expression and transcriptional activity.
- Demonstrated that co-transfection with MP1 leads to increased ER expression in E2-treated cells.

Reportable Outcomes:

1) Oral presentation at Midwest Regional Breast Cancer Symposium, July 2009. "Inhibition of MP1 expression induces apoptosis of ER-positive but not of ER-negative breast cancer cells". Abstract included in Appendix.
2) The results presented under Task 1 are currently being prepared for publication.

Conclusion: Our results have revealed a critical and previously unreported role for MP1 in the survival of ER-positive, but not ER-negative breast cancer cell lines. Blocking MP1 expression using siRNA leads to apoptotic cell death in ER-positive MCF-7, LCC9 and T47D cells, but not in ER-negative MDA-MB-231, SKBr3 and BT-549 cells. Overexpression of MP1 may also be toxic in ER-positive cells, since MCF-7 derivatives expressing ectopic MP1 are unstable and rapidly lose expression of the transfected MP1 gene upon passage. To our knowledge this is the first example of MP1 being involved in cell survival. Previous experiments in rat fibroblasts demonstrated that knockdown of MP1 led to a delay in cell spreading, but viability was apparently not affected (3).

The mechanism by which MP1 affects survival of ER-positive breast cancer cells is not known, but may be the result of alterations in ER level and/or function. In support of this hypothesis, MP1 knockdown leads to decreased ER levels, and MP1 overexpression leads to increased ER levels. Together, out data strongly suggest that MP1 may provide a novel target for the treatment of ER-positive breast tumors. Future experiments will further investigate the mechanism(s) by which MP1 regulates survival of ER-positive breast cancer cells, and those by which it regulates ER levels and function.

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3) Pullikuth A, McKinnon E, Schaeffer HJ, Catling AD. 2005. The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals. Mol Cell Biol. 25(12):5119-33.

Appendix/Supporting Data:

Figure Legends

Figures 1-6

Abstract from Midwest Regional Breast Cancer Symposium.

Figure 1. MP1 knock-down induces detachment and death of ER-positive breast cancer cells. Cells were transfected with control or MP1 siRNA according to the manufacturer's protocol (Dharmacon). Forty-eight hours later cells cultures were photographed, then both attached and floating cells were harvested and analyzed by cell counting and western blotting. A. MP1 knock-down using two different siRNA sequences causes detachment of MCF-7 cells. B. Western blots showing MP1 knocked-down in ER-positive cell lines MCF-7, LCC9, and T47D. Numbers represent the average decrease in MP1/actin ratio using 40 nM MP1 siRNA compared with control siRNA in 3 independent experiments. C. Left panel: Photographs of control and MP1 siRNA transfected cells at 48 hr showing rounding up of cells. Right panel: Both attached and floating cells were harvested and stained with trypan blue, and number of live and dead cells in each sample was counted. The results are expressed as the percentage of the total number of cells in each sample (mean ± SD, n=3).

Figure 2. MP1 knock-down does not cause detachment or death of ER-negative breast cancer cells. Cells were treated as described in the legend to Figure 1. A. MP1 protein was successfully knocked-down in MDA-MB-231, BT-549, and Sk-Br-3 cells. Numbers represent the average decrease in MP1/Actin ratio of cells transfected with 40 nM MP1 siRNA compared with control siRNA in 3 independent experiments. B. Left panel: Photographs of control and MP1 siRNA transfected cells. Right Panel: Both attached and floating cells were harvested and stained with trypan blue, and number of live and dead cells in each sample was counted. The results are expressed as the percentage of the total number of cells in each sample (mean ± SD, n=3).

Figure 3. MP1knockdown induces PARP cleavage in MCF-7 but not MDA-MB321 cells in a caspase dependent manner. A) MCF-7 and MDA-MB-231 cells were transfected with control or MP1 siRNA, then harvested at 48 h and analyzed for PARP cleavage by immunoblotting. B) MCF-7 cells were transfected with control siRNA or with MP1 siRNA in the presence and absence of the pan caspase inhibitor z-VAD-FMK. Cells were harvested at 48 h and analyzed by immunoblotting.

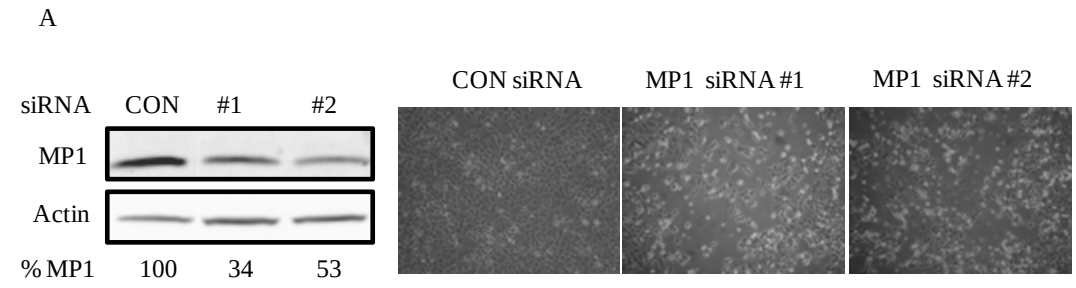
Figure 4. MP1 knock-down decreases transcriptional activity and expression of ER. A. MCF-7 cells were cotransfected with MP1 or control siRNA, along with an ERE-Luc reporter and a β-galactosidase control plasmid. Transfected cells were incubated in 5% CSS-IMEM for 24 h, then treated with 10 nM 17β-estradiol or vehicle for 8 h. Bars represent Luciferase/β-galactosidase activity (mean ± SD, n=5). B. Immunoblotting of ER in MCF-7 and T47D cells transfected with siRNA for 48 hours.

Figure 5. Co-immunoprecipitation of MP1 and ER in stably transfected cells. MCF-7 cells stably transfected with Flag-MP1 (A) or Flag-ER (B) were treated with CSS, CSS+E, CSS+Tam or CSS+ICI. In the case of Flag-ER cells, they were also treated +/- AP to induce Flag-ER expression. Cell extracts were prepared and immunoprecipitated with anti-Flag antibody. IP pellets were then resuspended in SDS-gel loading buffer and analyzed by SDS-PAGE followed

by immunoblotting with anti-MP1 and anti-ER antibodies. Total cell lysates were also analyzed for MP1, ER and actin levels.

Figure 6. Co-immunoprecipitation of MP1 and ER in transient co-transfections. Human 293 cells were co-transfected with expression vectors encoding Flag-MP1, ER, or control empty vectors as indicated. Cell extracts were prepared and immunoprecipitated with anti-Flag antibody. IP pellets were then resuspended in SDS-gel loading buffer and analyzed by SDS-PAGE and immunoblotting with anti-MP1 and anti-ER antibodies. Total cell lysates were also analyzed for MP1, ER and actin levels.

Figure 1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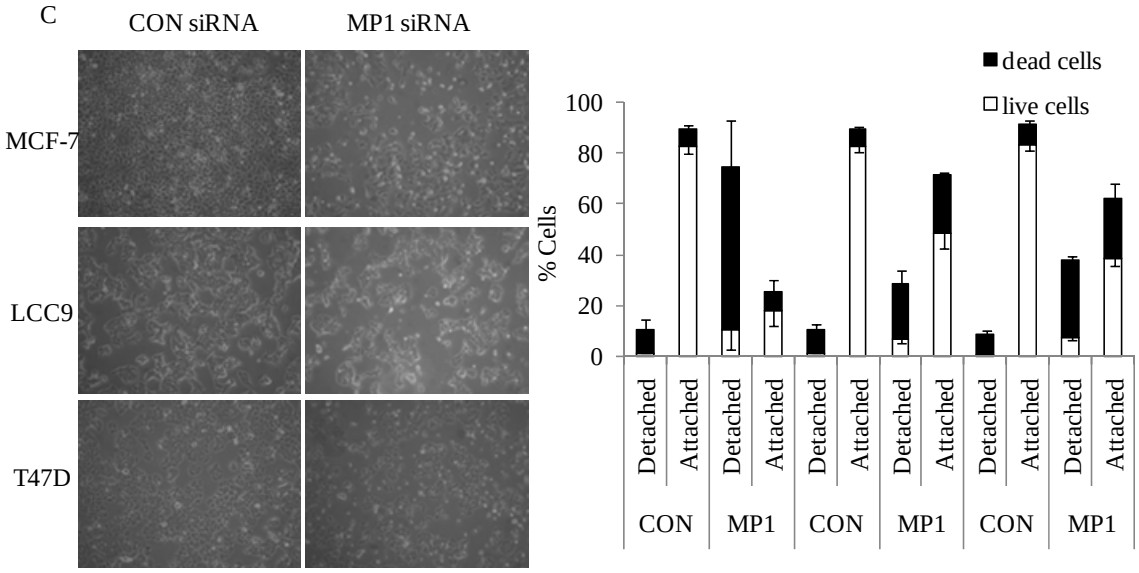
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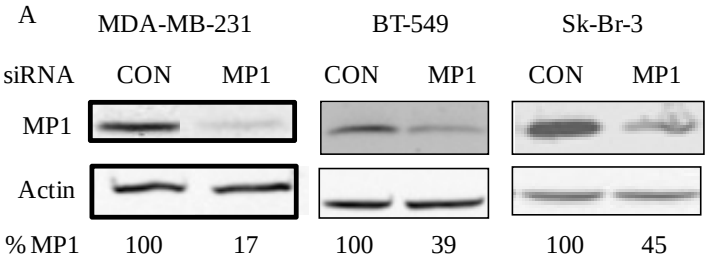
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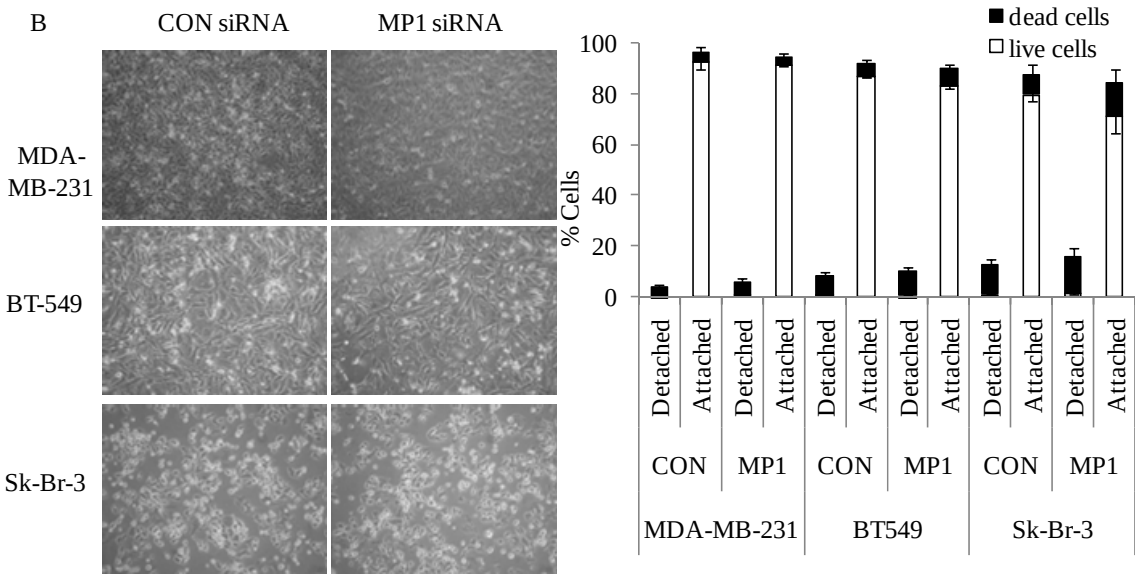
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Figure 3

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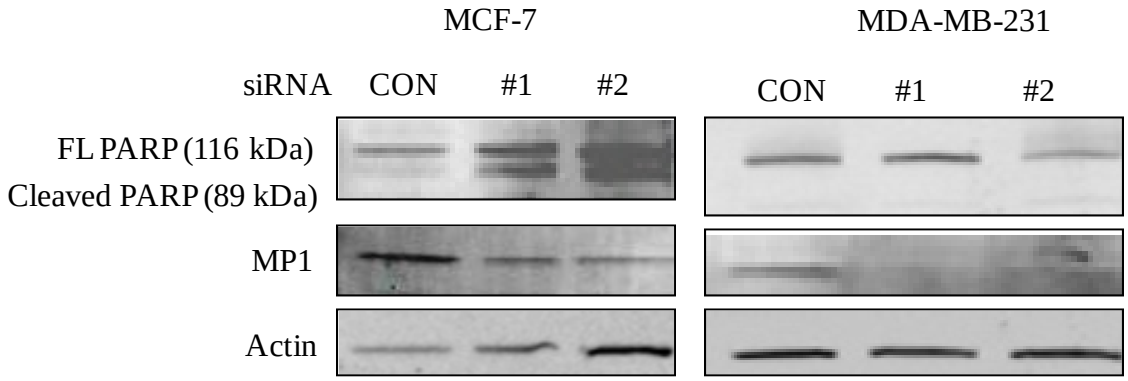
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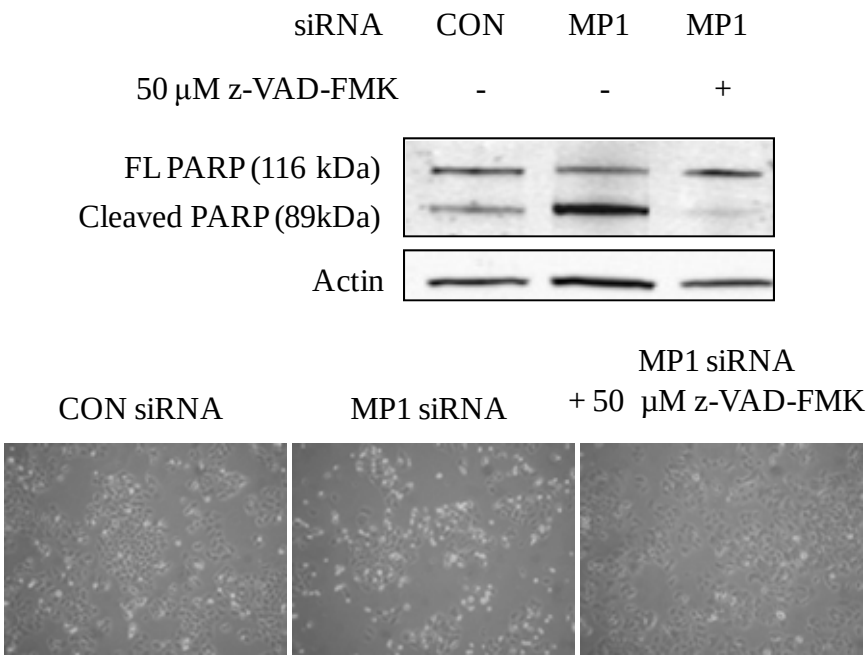
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Figure 4

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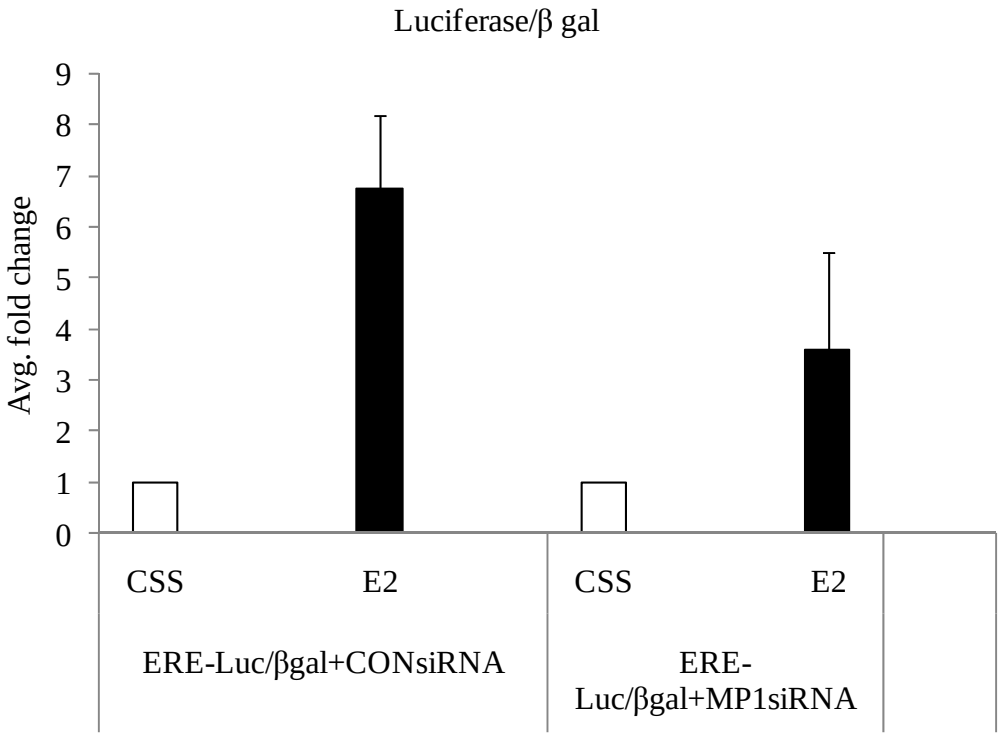
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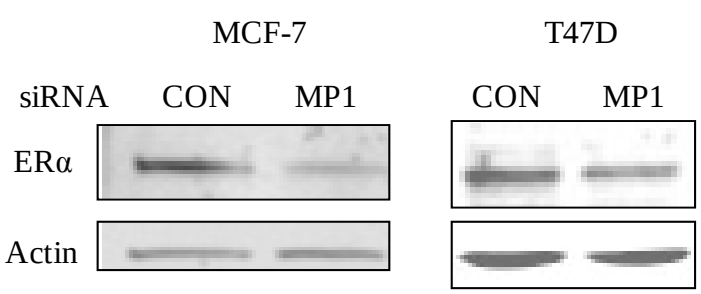
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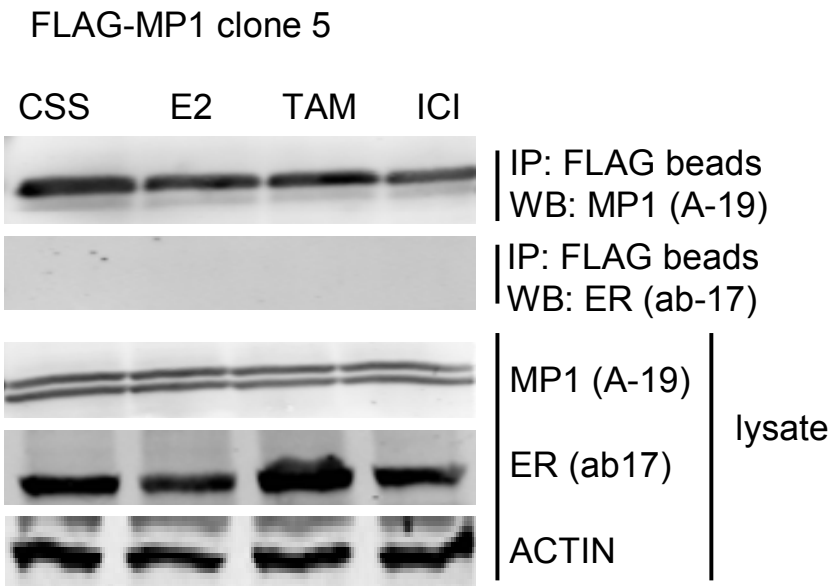
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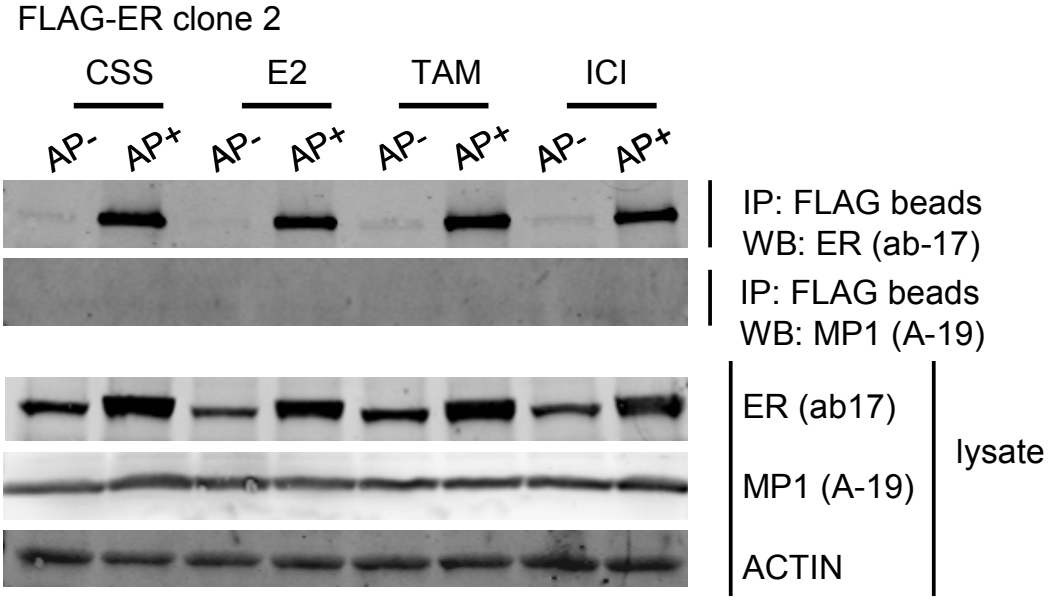
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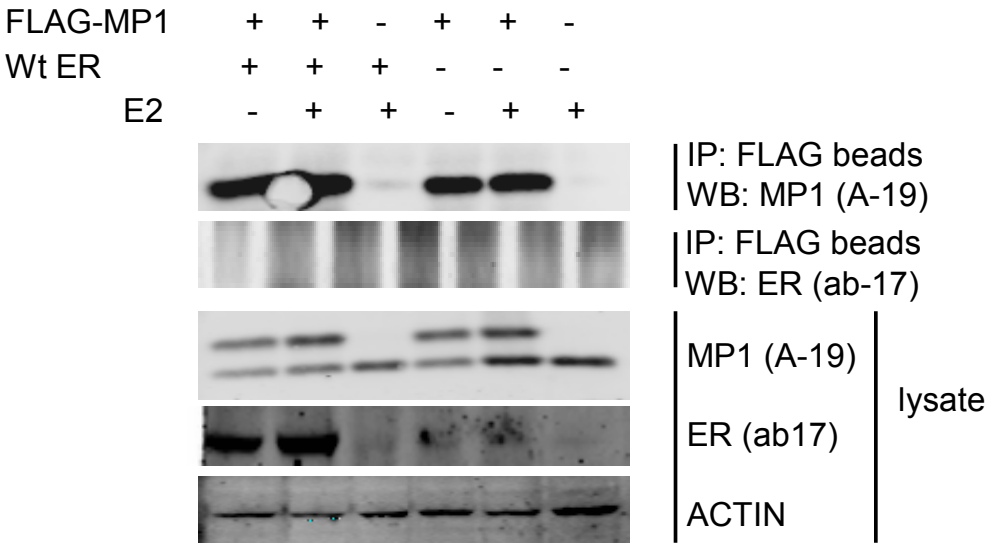
Figure 6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ACTIN
lysate

Inhibition of MP1 expression induces apoptosis of ER-positive but not of ER-negative breast cancer cells

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In approximately two thirds of breast cancer cases, the estrogen receptor alpha (ER) is a main regulator of tumor formation and growth. Unfortunately, as many as 30% of ER-positive breast tumors exhibit either innate or acquired resistance to endocrine therapies that target ER. The mechanisms by which resistance occurs are not completely understood, but involve activation of signaling networks that regulate cell proliferation and survival. Signaling molecules that ER interacts with represent potential diagnostic and/or therapeutic targets. MP1 is a scaffold protein that binds to MEK1 and ERK1 and that increases ERK activity when overexpressed. Despite being a widely expressed scaffold protein that may integrate multiple signaling pathways, the role of MP1 in mammary tumor cells remains unknown. Both ER and MP1 impact pathways that have been demonstrated to regulate proliferation and survival of breast cancer cells. We therefore hypothesized that MP1 might facilitate ER interactions with other signaling molecules and regulate its function. In this report we investigate the role of MP1 in two representative breast cancer cell lines: MCF-7 (ER-positive, estrogen-dependent, and anti-estrogen sensitive) and MDA-MB-231 (ER-negative). The effects of inhibiting MP1 expression, by transient transfection with siRNA duplexes were examined. After 48 hours, MP1siRNA-treated MCF-7 cells displayed evidence of cell death and this effect was absent in control siRNA treated MCF-7 cells and in both control and MP1siRNA treated MDA-MB-231 cells. Cell counting and trypan blue exclusion indicated that approximately 80% of MCF-7 cells rounded up and detached from the plate, with a majority of detached cells being trypan blue-positive, while MDA-MB-231 cells showed a minimal cell death effect. Protein immunoblotting confirmed that MP1 protein levels were successfully reduced in both cell lines. Cleavage of PARP-1 protein was detected in MCF-7 but not in MDA-MB-231 cells, suggesting that knockdown of MP1 causes an apoptotic cell death mechanism. Apoptosis of MP1siRNA treated MCF-7 cells was further confirmed using the pan-caspase inhibitor z-VAD-FMK which rescued the cell death phenotype.
These results were confirmed in additional breast cancer cell lines and demonstrate that MP1siRNA-induced cell detachment and cell death effects are specific to ER-positive breast cancer cells and may be, at least in part, dependent on estrogen signaling.